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Crossing the eukaryote-prokaryote divide

A ubiquitin homolog in the human commensal bacterium *Bacteroides fragilis*

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The resident microbiota of the human gastrointestinal (GI) tract is comprised of ~2,000 bacterial species, the majority of which are anaerobes. Colonization of the GI tract is important for normal development of the immune system and provides a reservoir of catabolic enzymes that degrade ingested plant polysaccharides. *Bacteroides fragilis* is an important member of the microbiota because it contributes to T helper cell development, but is also the most frequently isolated Gram-negative anaerobe from clinical infections. During the annotation of the *B. fragilis* genome sequence, we identified a gene predicted to encode a homolog of the eukaryotic protein modifier, ubiquitin. Previously, ubiquitin had only been found in eukaryotes, indicating the bacterial acquisition as a potential inter-kingdom horizontal gene transfer event. Here we discuss the possible roles of *B. fragilis* ubiquitin and the implications for health and disease.

Horizontal gene transfer (HGT) is a major driving force in the evolution of Eubacteria. For decades we have known that conjugation, transformation and bacteriophage-mediated transduction play important roles in the acquisition of novel functions. The advent of genomics, however, has illuminated the extent of HGT, for example, genomic comparison between three strains of *Escherichia coli*, the commensal K-12, enterohemorrhagic 0157 and uropathogenic CFT073, reveals only 39% shared genes, with much of the additional DNA in these strains acquired horizontally.¹

Interkingdom HGT appears to occur less frequently, with the most studied being the transfer of Ti plasmid DNA from *Agrobacterium tumefaciens* to plants. The barriers to interkingdom transfer appear greater, possibly because of differences in transcription and processing of transcripts, e.g., splicing. Bacterial to animal HGT has been documented primarily where endosymbiotic organisms inhabit an arthropod host, with *Wolbachia* sp. representing the largest number of reports.² Transfer of DNA from the Eukaryota to bacteria is less evident, with relatively few examples, such as the eukaryotic-like proteins encoded by *Legionella pneumophila* and a family of plant-like glycosyl hydrolases found in bacteria from the human gastrointestinal (GI) tract.^{3,4} Transfer of DNA from humans to bacteria, however, has been indicated by the presence of a fragment from the human long interspersed nuclear element L1 in the genome of the host-restricted pathogen *Neisseria gonorrhoeae*.⁵ This suggests that a close association between higher eukaryotes and their resident microbiota might facilitate HGT.

The open-ended culture system of the human gastrointestinal tract contains ~2,000 different species of bacteria. Within this diverse population, approximately 99% of the prokaryotic cells are anaerobes. The major groups of bacteria within the gut are represented by the phyla Bacteroidetes and Firmicutes.⁶ The Gram-negative strictly anaerobic *Bacteroides* spp predominate, as evidenced by their prevalence in the product of this culture system, faeces. *Bacteroides fragilis* is

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medically important because it is the most frequently isolated obligately anaerobic Gram-negative bacterium from human clinical infections, such as intra-abdominal, vaginal, pilonidal, perianal and brain abscesses.^{7,8} *B. fragilis* is also a common cause of anaerobic bacteraemia, with a potential mortality of up to 30%.⁹

Bacterial colonisation of the mammalian gut occurs after birth. The consumption of breast milk by neonates provides oligosaccharides that are substrates for fermentation by the genera *Bifidobacterium* and *Bacteroides*.¹⁰ The transition from passive immunity, associated with maternal milk, to innate and adaptive immunity occurs concurrently with the increased predominance of anaerobic bacteria in the GI tract. The important role that commensal bacteria play in ensuring correct development of the mammalian immune system is now becoming apparent, for example, polysaccharide A produced by some strains of *B. fragilis* alters the ratio of T helper cells and reduces production of IL-17 which aids in colonization of the GI tract mucosa.^{11,12} Other resident *Bacteroides* also activate additional developmental pathways, for example, *B. thetaiotaomicron* induces fucosylation of intestinal epithelial cell surfaces, promotes development of villus capillary networks and stimulates production of Angiogenin-4 from Paneth cells.¹³⁻¹⁵ The molecules responsible for modulating these latter effects are still unknown.

The genus *Bacteroides* represents approximately 10¹¹ cells/g of faeces in the lumen of the large intestine. Despite being a minor constituent of the faecal microbiota, *B. fragilis* is found at high cell densities in the mucosal layer adjacent to host epithelial cells in some individuals.¹⁶ This intimate association with host cells has the benefit of precluding invading pathogens from access to the gut epithelium, however, inappropriate host immune responses to the normal microbiota are thought to cause the tissue damage associated with inflammatory bowel disease (IBD).

During the genome sequencing of *B. fragilis* NCTC9343, we identified an 11kb region that has a lower GC content compared with the backbone sequence, suggesting acquisition by HGT. Within this region there are 12 predicted genes and

3 pseudogenes, one of which (BF3870) has homology to a fragment from a mobilization protein from the relic of a potential conjugative transposon in *B. thetaiotaomicron*, which reinforces the notion of horizontal acquisition. Surprisingly, within this region is a small open reading frame predicted to encode a homolog of eukaryotic ubiquitin that has 63% identity to human Uba52.¹⁷ The 76 amino-acid protein-modifier ubiquitin is highly conserved in the Eukaryota but, until now, has been considered to be absent from the Eubacteria.¹⁸ Ubiquitin has key roles in a multitude of cellular processes, including protein degradation, cell cycle progression, membrane protein endocytosis, intracellular trafficking, ribosome biogenesis, signal transduction, DNA repair, stress responses, chromatin-mediated regulation of transcription and antigen presentation.

To date, *B. fragilis* is unique in being the only bacterium to encode an identified ubiquitin homolog. The gene has evolved two additional features that differentiate the encoded protein from eukaryotic ubiquitin: first, *B. fragilis* ubiquitin (BfUbb) contains a signal sequence that directs it to the periplasm; second, BfUbb has lost the terminal glycine residues required for thioester bond formation with the catalytic cysteine residue in the eukaryotic E1 activating enzyme. Other residues that are important for interaction with the eukaryotic ubiquitination pathway, however, are conserved and BfUbb is capable of inhibiting the pathway in vitro.¹⁷ The presence of BfUbb in *B. fragilis*, and the additional features that have evolved, suggests it provides a selective advantage for the organism in the GI tract niche. This raises the question, "what is the function of *B. fragilis* ubiquitin?"

It is clear that many pathogenic bacteria, particularly during chronic infections, express virulence factors that interfere with ubiquitination to facilitate cell entry and modulate the innate immune response.^{19,20} *Yersinia pestis* uses a Type III secretion system to deliver YopJ to the cytoplasm of macrophages where it inhibits phosphorylation and subsequent ubiquitination of I κ B. Preventing release of I κ B inhibits activation of the pro-inflammatory response and assists in development of a systemic infection. A Type III

secretion system is also used by uropathogenic *E. coli* to inject the CNF1 protein into epithelial cells which subsequently deaminates Rho, Rac_{GTP} and CDC42 and so stimulates their ubiquitination and destruction. Proteolysis of these host proteins has the dual role of activating endocytosis of the bacterial cell and reducing the inflammatory response.²¹ Internalisation of *Salmonella enterica* typhimurium also requires modulation of Rac_{GTP} activity within macrophages. Deactivation of RacGTPase activity, by the *Salmonella* SptP protein, again leads to polyubiquitination and proteolysis of Rac which subsequently suppresses the inflammatory response and promotes cell recovery.²²

B. fragilis does not encode a Type III secretion system, so if BfUbb interacts with host proteins, how is it delivered to epithelial or other cells in the GI tract? Many Gram-negative bacteria produce outer membrane vesicles (OMV) which are released from the cell surface. *B. fragilis* produces OMV that are able to agglutinate red blood cells, but only if the OMV are coated in polysaccharides that represent the antigenically variable micro-capsule.²³ BfUbb is associated with concentrated supernatants that contain OMV, suggesting that transport of the protein to the periplasm allows its packaging and export via this route.¹⁷ OMV containing BfUbb could then deliver the cargo to epithelial cells either by membrane fusion or endocytosis. Whether the target of OMV is epithelial cells or phagocytic cells, such as M-cells, expression of BfUbb may provide *B. fragilis* with a selective advantage for colonization of the mucosa by suppressing the host immune system or interfering with other host-cell functions. Additionally, the "success" of *B. fragilis* as an opportunistic pathogen may be partially due to a ubiquitin-associated ability to evade destruction if the bacterium is engulfed by a macrophage.

Inappropriate immune responses to the resident GI tract microbiota contribute to inflammation of the mucosa and ultimately IBD. The pathways that recognize intracellular bacteria utilize receptors, such as NOD2, that bind to polyubiquitinated proteins, e.g., RIP2, as

scaffolds during initiation of the signaling cascade.²⁴ Mutations in NOD2 are associated with a predisposition to Crohn disease. Another allele associated with Crohn disease is ATG16L1, which is involved in intracellular clearance of invading bacteria by autophagy.²⁵ Targeting to autophagosomes involves binding of autophagic receptors, e.g., P62, to ubiquitinated proteins on the surface of intracellular bacteria.²⁶ A role for BfUbb in the genesis of Crohn's disease by interfering with ubiquitination of specific proteins has yet to be determined. Interestingly, inhibition of the E3 ubiquitin ligase, Itch, that ubiquitinates RIP2 causes inflammatory disease of the large intestine in mice.²⁷

The question remains as to the origin of *B. fragilis subsp.* Eukaryotic ubiquitin is highly conserved, however, the small number of viral genomes that encode ubiquitin homologs exhibit greater sequence diversity. The closest nucleotide similarity to *ubb* (216 bp) is found in the genome of a Migratory Grasshopper (*Melanoplus sanguinipes*) Entomopoxvirus (103/122 bp), with next closest being a Canaripox virus, another member of the Poxviridae. It can be hypothesized that the intimate association of *B. fragilis* with the human GI tract may have facilitated horizontal gene transfer from a eukaryotic virus, possibly associated with ingested material, to the *B. fragilis* genome.

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